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***C. elegans* vulval development as a model system to study the cancer biology of EGFR signaling**

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Abstract

Molecular genetic studies of *C. elegans* vulval development have helped to define an evolutionarily conserved signaling pathway from an EGF-like ligand through EGF-receptor, Ras and MAP kinase to the nucleus. Further studies have identified novel positive regulators such as KSR-1 and SUR-8/SOC-2 and negative regulators such as *cb1*/SLI-1. The many negative regulatory proteins might serve to prevent inappropriate signaling, and thus are analogous to tumor suppressor genes.

Introduction

Model genetic organisms have proven useful in the elucidation of cellular regulatory pathways and their normal physiological roles. EGFR is important during animal development in the control of cell growth, differentiation, metabolism, and migration, among many other biological responses. Here we review studies of EGFR signaling in *C. elegans* and mainly focus on its regulation during vulval development.

***C. elegans* biology and genetics**

To grow *C. elegans*, all one needs is an agar plate seeded with *E. coli* to serve as a growth medium, a wire pick to transfer animals from plate to plate for consistent log phase propagation or setting up a genetic cross, and an incubator to provide an environment of constant temperature (20°C) [1]. Animals can be maintained indefinitely in a frozen state and re-grown by thawing immediately. The short life cycle (three days per generation) makes experiments relatively rapid. One intriguing feature of *C. elegans* is its simple anatomy. Both sexes (male and hermaphrodite) have an essentially invariant cell lineage, and thus an almost constant cell number; the adult male has only 1031 somatic nuclei; the adult hermaphrodite has only 959 [2,3]. Forward or reverse genetics can both be used very convenient

in *C. elegans* to clone or characterize genes. There are three facts that make forward genetics practical in *C. elegans*. First, there are well-established genetic tools such as genetic markers and balancers distributed throughout the entire genome. Second, 80% of the genome are contained within cosmid pools and the rest 20% of the genome are covered by the YACs, which allows positional cloning to be efficiently done [4]. Moreover, a clone-based physical map of the *C. elegans* genome has been properly aligned with the existing genetic map. This allows genetic mapping to pinpoint a small physical region covered by a reasonable amount of cosmids and/or YACs that can be tested for rescuing the mutant phenotype in transgenic worm.

There are several advances that allow facile characterization of a gene function by reverse genetics in *C. elegans*. By microinjection of DNA into germline cytoplasm, we can efficiently transfer gene and stably maintain it as the extrachromosomal arrays from generation to generation, which allows us to repeatedly analyze the gene function in a specific genetic background after a single injection [5]. Recently, a technical breakthrough allows *C. elegans* experimenters to transiently knockout gene function as desired. Double-stranded RNA has been shown to robustly and specifically reduce, if not remove, gene expression in several species, including *C. elegans*, *Drosophila*, *Trypanosoma brucei*, and plants [6,7]. Stable knockout

of a single gene can also be performed by use of the chemical reagents. A desired deletion event is detected in a large pool of worms by means of PCR. The pool is consecutively subdivided into many small ones with PCR performed in each step till a single animal is isolated. Lastly, the essential completion of the *C. elegans* genome sequencing project gives us an opportunity to search for the homologs of genes of interest on a genome wide scale, and possibly to estimate the minimal set of players required to serve the biological functions of a signaling pathway.

The characterization of a signaling pathway does not proceed at the same pace among different systems. Therefore, a new component found in one system will boost the motivation to search for the homolog in other systems since we believe evolution favors the conservation of important functions. Until complete genome sequences are available, the failure to identify a homolog of gene of interest is not unusual. However, if we still fail to do so after the finish of genome sequencing, we should start to consider an alternative mechanism such that the function can be bypassed or there is redundancy.

Signal transmission by RTKs

All RTKs exhibit a similar molecular architecture and apparently are activated by a common mechanism [8,9]. A general mechanism for ligand-induced activation of RTKs has been established. Ligand binding to the extracellular domain induces receptor dimerization which in turn leads to activation of the catalytic protein kinase domain [10]. Autophosphorylation of tyrosine residues that are located in non-catalytic regions leads to generation of docking sites for SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains of signaling molecules [11]. These molecules include the adapter GRB-2, which leads to RAS activation, phospholipase C- γ (PLC γ), the tyrosine phosphatase SHP-2, RAS-GTPase activating protein, the regulatory subunit of phosphatidylinositol-3-OH kinase [11-13], and the proto-oncoprotein c-Cbl [14]. The association between proteins with SH2/PTB domains and pTyr serves as the initial step in the recruitment of an activated RTK signaling complex. Specific pTyr sites are recognized by distinct SH2 and PTB domains [15-19]. Interaction of RTKs with different substrates is thought to result in activation of distinct signaling pathways, thus producing distinct

cellular responses [8]. Excessive or inappropriate signaling from RTKs has been implied in many cases of malignant transformation [20,21]. Therefore, proper mechanisms must be employed to strongly control the signaling from RTKs to prevent ligand-independent activation and attenuate signaling after activation has occurred.

EGF RTK signaling is used multiple times during the normal development of *C. elegans*, mediating vulval induction, viability, male spicule development, hermaphrodite ovulation, and differentiation of the ventral uterus and posterior ectoderm [22-27]. Vulval induction was the first one discovered to be mediated by the EGF RTK signaling. The high resolution of scoring the phenotype of vulval induction makes it a very sensitive assay to identify the mutants and to reflect the relative strength of RAS-dependent signaling. All the other developmental events are also RAS-dependent, except for hermaphrodite ovulation, which will be discussed later.

EGFR signaling in vulval induction

The wild-type vulva is derived from three of six multipotential vulval precursor cells (VPCs), which receive an inductive signal from the anchor cell (AC) in the somatic gonad (Figure 1) [28]. The VPCs that form the vulva undergo three rounds of division and subsequent morphogenesis. VPCs that do not receive adequate signal from the AC divide only once and become part of a large syncytial epidermis that covers most of the worm. The inductive signal, LIN-3, is an EGF-like growth factor, produced by the anchor cell. LIN-3 activates the LET-23, a *C. elegans* homolog of EGF RTK, in the VPCs [29,30]. LET-23 activation initiates a signaling cascade that involves downstream effectors such as SEM-5, SOS-1, LET-60, LIN-45, MEK-2 and SUR-1/MPK-1 which are the nematode homologs of the mammalian Grb2 adaptor, the mSOS guanine nucleotide exchange factor, the Ras GTP-binding protein, the Raf serine/threonine kinase, the MAPK kinase and the MAPK, respectively [31-39] (C. Chang and P. Sternberg, unpublished). *Loss-of-function (lf)* mutations in any of the signaling proteins in the LET-23 RTK pathway result in less than three VPCs undergoing vulval differentiation, whereas *gain-of-function (gf)* mutations result in more than three VPCs undergoing vulval differentiation.

Apart from the main RAS-dependent signaling pathway, there are two accessory proteins identified in

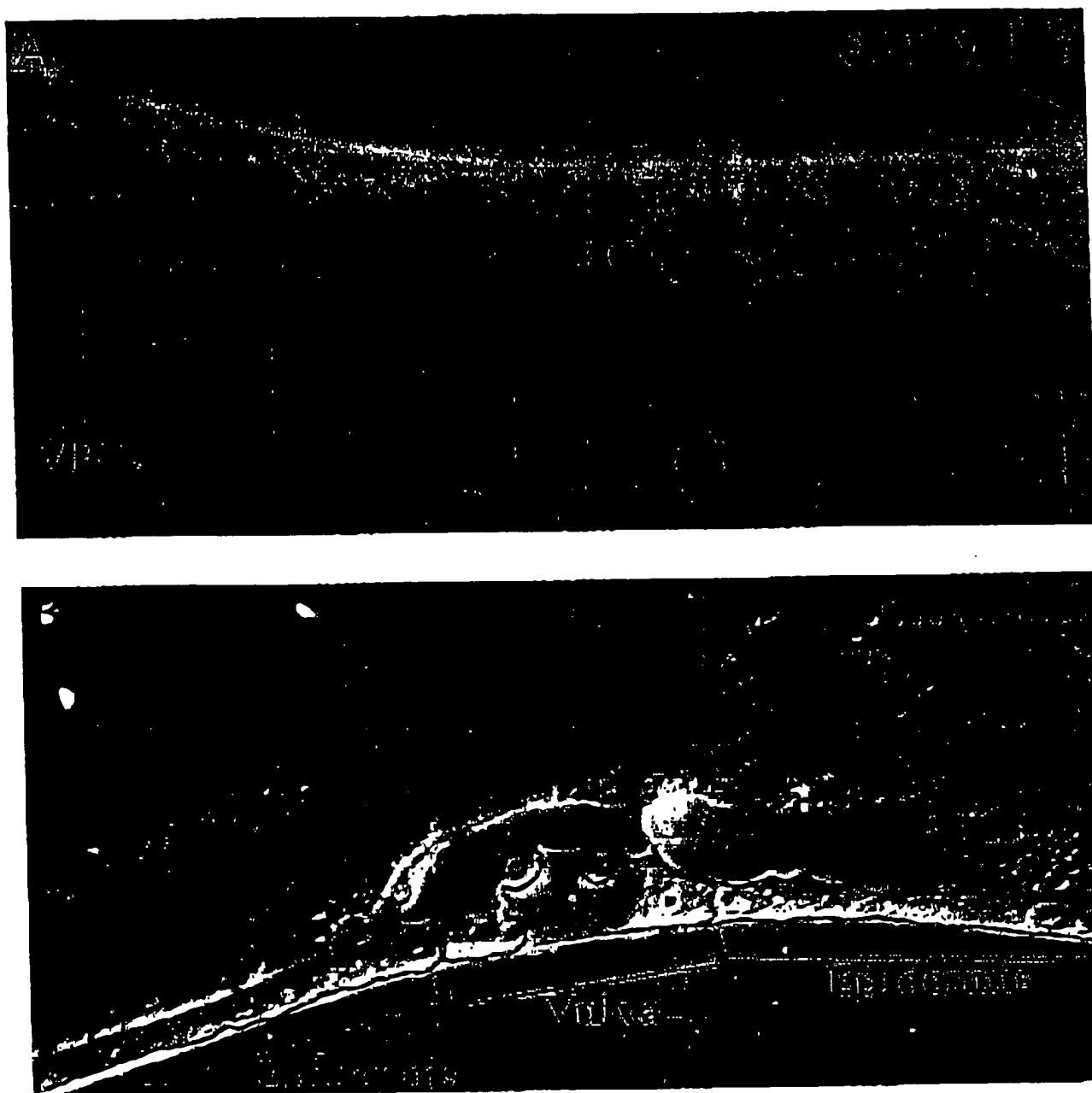


Figure 1. A. *C. elegans* vulva development occurs postembryonically. Six ventral midline-located VPCs are competent to adopt any of three cell fates (1°, 2° and 3°). In the early third larval (L3) stage, upon being induced by a gonadal AC-produced signal, the AC-closest VPC adopts 1° fate, which gives rise to eight descendants, while the adjacent VPCs adopt 2° fate, which gives rise to seven descendants. Further away, the three VPCs adopt 3° non-vulval fate, due to insufficient receiving of the inductive signal. B. After the last round of cell division, cells of a canonical 1° lineage move dorsally and detach from the ventral cuticle. By the mid-L4 stage, they form a symmetric arch and separate antero-posteriorly and left-right to create a hole so that eggs can pass through it. The 2° lineage is asymmetric; the proximal cells detach and migrate dorsally and the AC-distal cells keep attaching to the ventral epidermis. By the mid-L4 stage, they form a characteristic structure.

a screen for the extragenic suppressors of the G13E-activated mutant *let-60 ras*. *ksr-1*, encodes a conserved Raf-related serine/threonine kinase, which has been shown to act downstream of or in parallel to *let-60 ras* and upstream of *lin-1*, a putative ETS transcription factor [40,41]. *sur-8/soc-2*, which encodes a conserved Ras-binding protein with leucine-rich repeats, appears to act downstream of or in parallel to *let-60 ras* but upstream of *lin-45 raf* [42,43]. Mutations in either loci do not affect normal vulval induction. However, a *sur-8/soc-2* mutation significantly synergizes with a *loss-of-function* mutation in *ksr-1*, suggesting that they are collectively essential in the presence of the main RAS-dependent signaling pathway.

A mutant of *ptp-2*, another positive effector for *let-23*-mediated vulval signaling, identified by a PCR-based screen for transposon-mediated deletion, appears to be not essential for wild-type vulval induction [44]. SH2 domain-containing PTP-2 has a predicted protein structure similar to the mammalian SHPs and *Drosophila* Corkscrew. *Loss-of-function* in *ptp-2* suppresses the multiple vulva (Muv) phenotype induced by a *loss-of-function lin-15* mutation, and an activated *let-23 EGFR* or *let-60 ras* mutation. The role of *ptp-2* function in the vulval signaling pathway is not clear.

Three negative regulators of LET-23 mediated vulval differentiation were isolated as extragenic suppressors of *let-23(lf)* and have been cloned. UNC-101 is a *C. elegans* homolog of mammalian AP-47 clathrin medium chain protein [45]. SLI-1 is a *C. elegans* homolog of mammalian proto-oncoprotein c-Cbl [46,47]. GAP-1 is a *C. elegans* homolog of mammalian Ras-GTPase activating protein (RasGAP) [48]. One negative regulator was isolated as an extragenic enhancer of *sli-1(lf)* and has also been cloned. ROK-1 is a non-receptor protein tyrosine kinase (PTK) related to the Ack sub-family (N. Hopper, J. Lee and P. Sternberg, unpublished). Mutations in each of these four negative regulators are silent by themselves. However, in combination with mutations in any other loci defining negative regulators results in a Muv phenotype (G. Jongeward, N. Hopper, C. Chang, C. Lacenere and P. Sternberg, unpublished).

Yet another set of apparently redundant negative regulators are known. Genetically, there are two classes of the so-called synthetic multivulva (synMuv) genes, class A and class B. Class A genes include *lin-8*, *lin-15A*, *lin-38*, and *lin-56* and class B genes include *lin-9*, *lin-15B*, *lin-35*, *lin-36*, *lin-37*, *lin-51*, *lin-52*, *lin-53*, *lin-54*, and *lin-55* [49–51]. Animals containing mutations in only class A or class B genes are wild-type,

whereas animals carrying mutations in both classes of genes are Muv. Two class B synMuv genes, *lin-35* and *lin-53*, have been shown to encode proteins similar to Rb and its binding protein RbAp48. *lin-15*, a complex locus contained two mutable genetic activities (A and B), encodes two non-overlapping transcripts involved in the negative regulation of vulval induction [52,53]. The predicted proteins from *lin-15A* and *lin-15B* transcripts are not similar to any identified proteins. Mutations in either *lin-15A* or *lin-15B* are phenotypically wild-type and a Muv phenotype can be induced only if both activities are removed. There seems to be a fundamental difference between synMuv genes and the negative regulators discussed above. The negative effects of class A synMuv genes are strictly redundant to class B synMuv genes, whereas the negative effects of those negative regulators mentioned before are partially redundant among them (Figure 2). It is unclear how, mechanistically, the synMuv proteins regulate LET-23–Ras signaling.

Recently, another negative regulator, *sur-5*, was identified in a screen for the extragenic suppressors of a dominant negative (*dn*) *let-60 ras* allele [54]. *sur-5* encodes a novel protein with one potential ATP or GTP binding motif and two potential AMP binding motifs. Mutations in *sur-5* have no obvious phenotypes of their own and do not suppress *loss-of-function* mutations in the *let-23 EGFR* signaling pathway either upstream or downstream of *let-60 ras*, including *let-60 ras* itself. It is interesting that *sur-5* mutations specifically suppress one of two subsets of *let-60 ras (dn)* alleles. Since all the *let-60 ras (dn)* mutations are suppressed by the *let-60 ras (gf)* mutation in trans-heterozygotes [31,32,55], it is believed that the toxic effect of a dominant negative LET-60 RAS protein is due to the competition with wild-type LET-60 RAS protein for the upstream guanine nucleotide exchange factor (GNEF) instead of downstream effectors. These results raise the possibility that there might be at least two different activators for LET-60 RAS and only one of them is titratable by the subset of *let-60 ras (dn)* mutant proteins that is suppressed by the *sur-5* mutations.

Reciprocal EGF signaling

lin-3 EGF is first produced by the gonadal AC to induce VPCs to divide and differentiate into vulval tissue. After vulval induction, *lin-3* EGF is also expressed in the 1° vulval lineage (Figure 3) [27]. EGF signaling

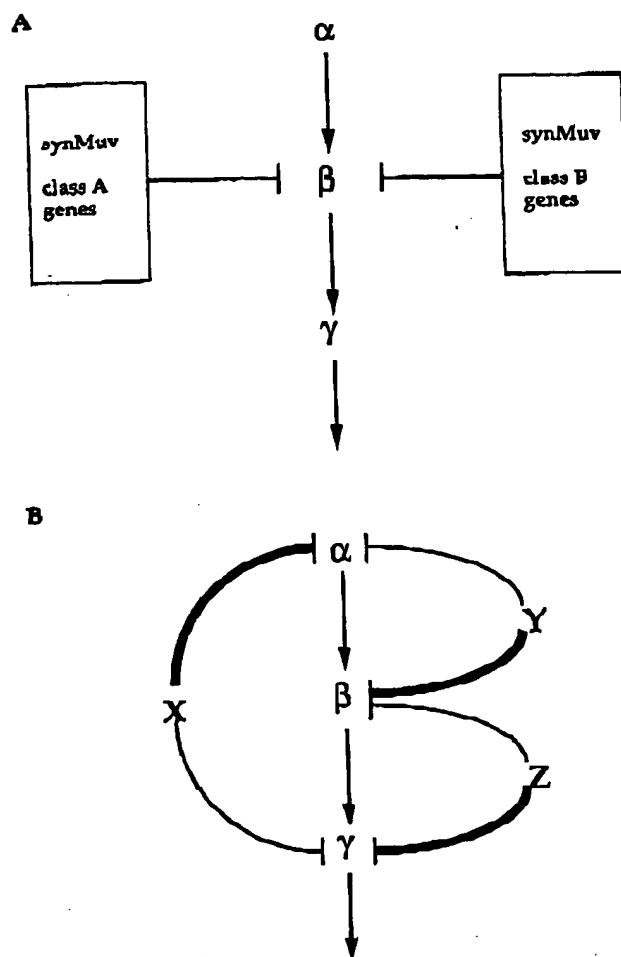


Figure 2. A. A hypothetical regulatory circuit operated by the *synMuv* genes. Arrows indicate a positive effect and bars indicate a negative effect. There is strictly a redundancy between *synMuv* class A genes and class B genes in negatively regulating the *let-23* *EGFR* signaling pathway. Only mutation(s) in class A genes combined with mutation(s) in class B genes will result in the excessive signaling and the Muv phenotype. B. A hypothetical regulatory circuit operated by *slf-1*, *unc-101*, *rok-1* and *gap-1*. Arrows indicate a positive effect and bars indicate a negative effect with thickness of the line reflects strength of the effect. X, Y and Z represent the negative regulations provided by these signaling regulators. The inhibitory effects performed by any two of them are partially redundant such that mutations in any two of them will lift the restraint on one signaling node, that results in the excessive signaling and the Muv phenotype.

from cells of the 1° vulval lineages to a subset of ventral uterine cells of the gonad is required for the specification of uterine uv1 cells, which form part of the uterine-vulval connection. While mutation of *SLI-1* restores most of the reduced *LET-23* signaling in the

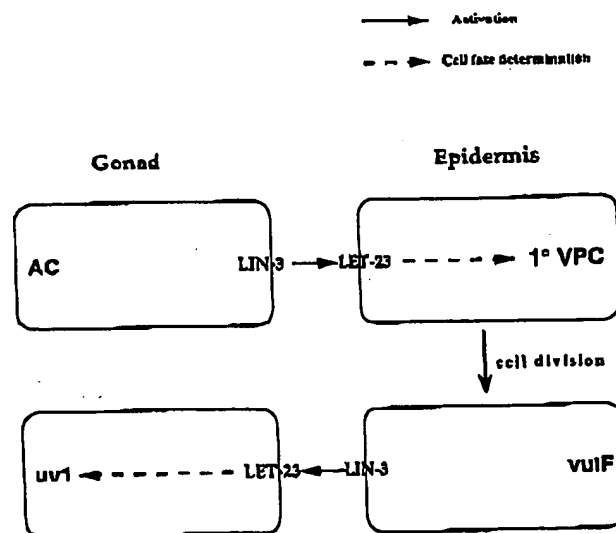


Figure 3. Reciprocal EGF signaling from the vulva specifies the uv1 cell fate. *vulF*, the granddaughter of the 1° VPC, is the dorsal-most 1° vulval progeny. *uv1*, the ventral-most uterine cell, makes the direct contact with *vulF* during the development of the uterine-vulval connection.

vulva caused by *let-23(lf)* mutation, the defects in uv1 cell fate are only partially rescued. This specificity most likely comes from the quantitative difference of *slf-1* usage. In contrast, a *gap-1(lf)* mutation suppresses the vulval but not the uv1 defect displayed by the same *let-23(lf)* mutation, suggesting that *LET-23*-mediated signaling is not regulated by *GAP-1* in the presumptive uv1 cells.

EGFR signaling in ovulation

LET-23 function in the somatic gonad is required for ovulation in hermaphrodite and is independent of *RAS*. While mutations in *lfe-1/irr-1* and *lfe-2* suppress the ovulation defects associated with *let-23(lf)* mutant, mutations in the components of *RAS* pathway do not [25]. *lfe-1/irr-1* encodes a *C. elegans* inositol (1,4,5) trisphosphate receptor (IP3R) and *lfe-2* encodes an inositol (1,4,5) trisphosphate-3-kinase (IP3K). Since a *gf* mutation in *lfe-1/irr-1* and a *lf* mutation in *lfe-2* rescue the ovulation defects of a *let-23(lf)* mutant, *lfe-1/irr-1* is inferred as a positive effector of *LET-23* signaling whereas *lfe-2* a negative regulator. The comparison of *C. elegans* vulval development and hermaphrodite ovulation thus provides an excellent example of how an RTK evokes its tissue specificity by deploying the distinct signaling cascades. The *let-23(gy97)* mutant

animals are defective in all *let-23*-mediated phenotypes except for the hermaphrodite ovulation [56]. This mutation deletes the last 56 amino acids of the receptor which include the only three tyrosines (pTyr sites 6, 7 and 8) in the receptor carboxy terminal tail which, if phosphorylated, would create SH2 binding sites matching the consensus binding site for the SEM-5 SH2 domain. Consistent with these findings, in the *let-23(null)* background, none of the site deleted by the *let-23(sy97)* mutation is able to confer fertility and *let-23* construct bearing either only site 4 or site 5 confers full or 15% fertility [57]. Although *let-60/mek-2/mpk-1* cascade responses to *let-23* activation in every tissue where *let-23* activity is required for the proper development, the inductive signal used for *let-60/mek-2/mpk-1*-mediated germline meiotic cell cycle progression is still unknown.

Table 1 shows genes of *let-23 EGFR* signaling pathway identified in *C. elegans* either by sequence homology search or genetic mutation. Many sequence homology searches are based on information obtained from the biochemical studies in mammalian systems where a putative role in EGF RTK signaling was implied. Some of the SH2 and PTB domains of signaling molecules, although found to be associated with the activated EGF RTK, are not formally demonstrated regarding their functional relevance in the regulation of signaling (effectors or regulators). For those having a corresponding mutants, the phenotypes observed obviously display their physiological involvements in the EGF RTK signaling. Mutants may be recovered by either a classical genetic screen or a PCR-based screening for deletions. Double-stranded RNA interference is also used to identify some of the signaling components or to confirm the mutant phenotypes.

EGFR signaling is conserved during evolution

Although the cellular interpretation of signaling are context-dependent, the RAS-dependent EGF RTK signaling pathways are well conserved at the level of their mechanistic aspects during metazoan evolution. As a result, a core set of players required for the execution of the signaling share high similarity among species in terms of their functions, protein structures, and one-dimensional amino acid sequences. Differences obtained from evolution predominantly reside in the redundancy of signal transduction and the complexity of its regulation. In *C. elegans*, all known effects of LIN-3 are mediated through LET-23 and

there is no evidence that LET-23 has additional ligands apart from LIN-3. In the VPCs, upon the binding by LIN-3, LET-23 is presumably autophosphorylated thereby creating docking sites for the proteins containing SH2 or PTB domains, such as SEM-5. Based on results with the mammalian proteins, SOS-1 likely constitutively binds SEM-5 via its proline-rich domain. The activated receptor-bound SEM-5 therefore brings SOS-1 close to the membrane-anchored LET-60 and that catalyzes the release of GDP from LET-60 and allows its loading with GTP.

Mechanisms of negative regulation of LET-23 signaling

Unlike the intensively studied mechanistic aspects of signaling transduction by Ras-dependent RTKs, the molecular mechanisms of their negative regulation are still largely unclear. Generally, there are two ways to negatively regulate signaling. One is to regulate the basal activity of signaling in the absence of the ligand. The other is to attenuate or desensitize the signaling after it occurs. RasGAP inhibits the Ras activity by stimulating its intrinsic GTPase activity, thereby increases the level of GDP-bound, inactive Ras [58]. Apart from the catalytic domain, *C. elegans* GAP-1 does not display any significant similarity to other proteins in the database [48]. SH2 and SH3 domains, that are found in the mammalian p120 RasGAP, are not present in the *C. elegans* GAP-1. *C. elegans gap-1* acts to inhibit the vulval induction since *loss-of-function* mutation in the *gap-1* suppresses the vulvaless (Vul) phenotype associated with *loss-of-function* mutations in the *let-23 EGFR/let-60 ras* mediated signaling pathway. It remains an open question whether *C. elegans* GAP-1 is a constitutive inhibitor of RAS and not dependent upon the ligand/receptor.

sli-1 has been shown to inhibit the *let-23 EGFR*-mediated vulval signaling upstream of *let-60 ras* by genetic suppression analysis [46]. So far, there is no report from biochemical studies that shows an interaction between c-Cbl and mSOS, but many reports demonstrated the physical association between c-Cbl and Grb2 or c-Cbl and activated EGFR. c-Cbl binds to Grb2 through its polyproline motifs and colocalizes with EGFR via its diverse SH2 domain, which is dependent upon ligand activation. The sequence of D-Cbl, a *Drosophila* homolog of c-Cbl, ends immediately C-terminal to the RING finger motif [14,59]. Although D-Cbl lacks polyproline motifs, expression

Table 1. Genes involved in *let-23* EGFR-mediated signaling

	Genes	Genetic screens used to isolate the mutant alleles	Available mutations	Linkage groups	Nature of proteins
Defined by mutants					
Ligand	<i>lin-3^Δ</i>	1,†	<i>lf, gf</i>	IV	EGF
Receptor	<i>let-23ⁱ</i>	1 and 2, 3	<i>lf, gf</i>	II	EGFR
Positive effectors	<i>sem-5</i>	2 and 4	<i>lf</i>	X	Grb2 adaptor
in vulva induction	<i>sos-1</i>	2 and 4 and 5	<i>lf</i>	V	RasOEF
	<i>let-60^Δ</i>	2 and 6, 3 and 7, 2	<i>lf, gf, dn</i>	IV	Ras-like GTPase
	<i>ptp-2</i>	8	<i>lf</i>	II	SHPs
	<i>sur-8/soc-2ⁱ</i>	4 and 9	<i>lf</i>	IV	leucine-rich repeats
	<i>ksr-1</i>	9	<i>lf</i>	X	Raf-related kinase
	<i>lin-45ⁱ</i>	2 and 9, 10, 10	<i>lf, gf, dn</i>	IV	Raf Ser/Thr kinase
	<i>mek-2ⁱ</i>	9, 10, 10	<i>lf, gf, dn</i>	I	MAP kinase kinase
	<i>mpk-1/sur-1ⁱ</i>	9	<i>lf</i>	III	MAP kinase
	<i>lfe-1/lir-1</i>	11	<i>gf</i>	IV	IP3R
in ovulation					
Negative regulators	<i>lin-15</i>	3	<i>lf</i>	X	Novel protein
in vulva induction	<i>sli-1</i>	5 and 7	<i>lf</i>	X	Proto-oncogene c-Cbl
	<i>rok-1</i>	12	<i>lf</i>	IV	Non-receptor PTK
	<i>unc-101</i>	7	<i>lf</i>	I	AP47
	<i>gap-1</i>	7	<i>lf</i>	X	RasGAP
	<i>sur-5</i>	13	<i>lf</i>	X	Novel protein
in ovulation	<i>lfe-2</i>	11	<i>lf</i>	I	IP3K
Defined by sequence similarity					
	Dos				Daughter of sevenless
	PLC γ				Phospholipase C γ
	Nck				SH2/SH3 adaptor
	Crk				SH2/SH3 adaptor
	PI3K p110				Catalytic subunit of PI3 kinase
	PI3K p85				Regulatory subunit of PI3 kinase
	Cnk				Connector enhancer of KSR

1. Direct screening for the Vul mutants from the wild-type animals.

2. Extragenic suppressors of the Muv phenotype of *lin-15(lf)* mutants.

3. Direct screening for the Muv mutants from the wild-type animals.

4. Extragenic suppressors of the 'Clear' phenotype of *clr-1(lf)* mutants. *clr-1* encodes a *C. elegans* homolog of receptor tyrosine phosphatase. 'Clear' phenotype indicates the clarity of cell boundaries, reflecting a hyperactive FGF receptor signaling [34].

5. Double-stranded RNA interference.

6. Intragenic revertants of *let-60(dn)* mutants.

7. Extragenic suppressors of the Vul phenotype of *let-23(lf)* mutants.

8. PCR-based screening for transposon-mediated deletion mutant.

9. Extragenic suppressors of the Muv phenotype of G13E-activated *let-60 ras* mutants.

10. Mutations corresponding to either mammalian or *Drosophila* changes were created by site-directed mutagenesis and the transgenes are put under control of the heat shock promoter.

11. Extragenic suppressors of sterility phenotype of *lin-3(lf)* mutants.

12. Extragenic enhancers of the 'pre-Muv' phenotype of *sli-1(lf)* mutants. 'pre-Muv' represents a state where an excessive *let-23 EGFR* signaling does not overcome the threshold to cause a Muv phenotype but is sensitive to the fluctuation of the signaling.

13. Extragenic suppressors of the Vul phenotype of *let-60(dn)* mutants.

*When a genetic *gain-of-function* mutation is not available, increase of gene dosage by the wild-type transgene mimics the *gain-of-function* phenotype. To give an effect by overexpressing transgene, the endogenously expressed-gene products must be relatively limited when compared to other proteins.

^ΔMultiple copies of transgenes produce the dominant Muv phenotype, suggesting that the quantities of their gene products are normally limited in the vulval signaling.

ⁱMultiple copies of transgenes do not produce the dominant Muv phenotype, suggesting that the quantities of their gene products are not normally limited in the vulval signaling.

of the *sevenless* enhancer-driven D-Cbl significantly abolishes the development of R7 photoreceptor neuron, suggesting that polyproline motifs might be evolutionarily dispensable for the function of c-Cbl-like proteins in regulating the EGFR signaling. Although our preliminary results show that the polyproline motifs are not essential for *sli-1* function in the vulva, the presence of polyproline motifs do help to achieve the full activity of *sli-1* (C. Yoon, C. Chang and P. Sternberg, unpublished).

Our current models for the *sli-1* functions propose two roles. The major role of *sli-1* might be to attenuate signaling after activation has occurred. This model is supported by the evidence that c-Cbl-like proteins only associate with EGFR in an activation-dependent manner. Indeed, by analyzing the systematically mutagenized *let-23* constructs containing substitutions in the carboxyl-terminal tyrosine residues, we have identified one inhibitory phosphotyrosine residue, which can overcome the negative regulation by *sli-1* when it is mutated (C. Chang, N. Hopper and P. Sternberg, unpublished). The observation is strengthened by a recently solved crystallographic structure of c-Cbl complexed to a tyrosine-phosphorylated inhibitory site of protein tyrosine kinase ZAP-70 [60]. The interaction is mediated by a divergent SH2 domain of c-Cbl, which is conserved in SLI-1, and a phosphotyrosine of ZAP-70, which is in a similar amino acid context to the one we identified in LET-23. The minor role of *sli-1* might be to regulate the basal activity of signaling in quiescent state by competing with SOS-1 for the binding of SEM-5, thereby decreasing the chance that the spontaneously activated receptor recruits the SOS-1-bound SEM-5 in the absence of ligand. Upon stimulation by the inductive signal, SLI-1 molecules might switch to bind the tyrosine-phosphorylated receptors, thereby relinquishing the interference on the binding between SOS-1 and SEM-5. While *sli-1* negatively regulate the *ras*-dependent vulval differentiation, it does not affect the *lfe-1*-dependent hermaphrodite ovulation. The simplest interpretation is that there is a tissue-specific expression or activation of *sli-1*. Alternatively, the *ras*-dependent and *lfe-1*-dependent signaling cascades might have fundamental differences such that *sli-1* can only inhibit one signaling cascade but not the other. For example, *ras*-dependent and *lfe-1*-dependent signaling cascades are elicited by distinct phosphotyrosine residues of *let-23* EGFR, which could have different steric orientations. While the activated receptor-bound *sli-1* is able to execute its negative regulation in one steric orientation, it fails in the other.

A localized *let-23* EGFR-dependent negative regulation has been proposed previously since a *reduction-of-function* mutation in *let-23* EGFR, which results in the mislocalized receptor, causes a Muv phenotype in a *sli-1* or *gap-1* mutant background [46] (G. Jongeward and P. Sternberg, unpublished). Genetic mosaic analysis showed that the presence of *let-23* EGFR in the proximal VPCs inhibits the vulval induction in their more AC-distal VPCs [48]. A possible mechanism for this observation is that the AC-proximal VPCs bind and sequester the AC-produced ligand LIN-3 EGF, thereby preventing the ligand from reaching more distal VPCs. In this view, the sequestering mechanism serves to down regulate the ligand after it activates the receptor in the AC-proximal VPCs.

The negative regulation of *let-23* EGFR-mediated vulval signaling by synMuv genes is ligand-independent: gonad ablation does not affect the Muv phenotype induced in the *lin-15AB* mutant background [53,61]. Mutations in *let-23* EGFR and its downstream effectors suppress the Muv phenotype induced by *lin-15* mutations. Taken together, these data suggest that *lin-15* acts upstream of *let-23* EGFR but downstream of or in parallel to *lin-3* EGF. Although mosaic analysis suggests that *lin-15* functions in the hyp7 epidermis and hence regulates *let-23* signaling in the VPCs in a cell non-autonomous manner [62], the LIN-15 proteins are present in the VPCs nuclei (L. Huang, J. DeModena and P. Sternberg, unpublished). Indeed, *lin-35* Rb and *lin-53* p48, two loci previously assigned to the class B synMuv pathway, have been shown to express in the VPCs nuclei [51]. If *lin-15* does function in nuclei of the VPCs to negatively regulate *let-23*-mediated vulval signaling as opposed to downstream nuclear targets, there are two possible targets for *lin-15* action, LET-23 EGFR and its activators. It is less likely that *lin-15* only acts to inhibit the expression of *let-23* since overexpression of *let-23* does not cause a Muv phenotype as by *lin-15* mutations [29] (G. Lesa and P. Sternberg, unpublished). The combinatorial effects on the expression of *let-23* and its activators might, ultimately, explain *lin-15* function in the VPCs.

Conclusions

1. *C. elegans* LET-23-mediated vulval induction requires *C. elegans* homologs of EGFR, Grb-2, mSOS, Ras, Raf, MAPK kinase, and MAPK, which are conserved during metazoan evolution.

2. New positive effectors and negative regulators of EGFR-mediated signaling have been identified and characterized by *C. elegans* molecular genetics.
3. One way in which EGFR has tissue-specificity is to use distinct signaling cascades in different tissues.

Key unanswered questions

One way to modulate the strength of signaling is by controlling the production and presentation of the ligand. Identification of tissue-specific transcriptional regulators and proteases for the ligand LIN-3 will be required to understand this aspect of regulation. RAS activity is necessary not only for the vulval induction but also for the subsequent patterning events [27]. The amplitude and duration of RAS-dependent EGFR signaling required for proper vulval development is unclear. To address such a question, it is essential to develop an assay to monitor the signaling activity during animal development. Although binding to the EGFR seems to be necessary for the negative regulation by *sli-1*, the mechanisms by which *sli-1* exerts its negative effects are not well understood. Since *sli-1* mutation fails to complement a *gap-1* mutation (C. Chang and P. Sternberg, unpublished), *sli-1* and *gap-1* might act in a linear pathway where *sli-1* exerts its negative effects by directly or indirectly activating *gap-1*. This hypothesis is supported by the only weak synergy between *sli-1* and *gap-1* mutants (C. Chang and P. Sternberg, unpublished). How does SOC-2/SUR-8 fit into the RAS signaling? If interaction between SOC-2/SUR-8 and RAS is necessary for the normal function of signaling, SOC-2/SUR-8 could be either another effector for RAS or a modulator to facilitate the association between RAS and effectors. PTP-2 functions as a supporting component during vulval development. How is it integrated into the main signaling framework? The identification of PTP-2 substrates should allow us to understand its contributions to the signaling.

One aspect of vulval development not explicitly considered here might prove especially relevant to tumor cell biology. After inducing the vulva, the gonadal anchor cell invades the vulval epithelium, initiating the hole that will ultimately connect the lumen of the uterus to that of the vagina [63,64] (R. Palmer, D. Sherwood, K. Tietze and P. Sternberg, unpublished). This process appears analogous to tumor cell invasion during metastasis, and might provide a genetic model system to investigate invasion.

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